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Modulator of Inflammation

The present invention is concerned with a specific molecular mechanism by which inflammation and susceptibility to infectious disease may be genetically regulated, more specifically with a molecular mechanism modulating the expression of tumour necrosis factor-α (TNF-α). In particular, the invention relates to two novel DNA binding proteins which bind specifically to DNA target sites in the TNF-α promoter region, to assays based on this protein/DNA interaction and to a method of screening for susceptibility to inflammatory disease based upon screening for a polymorphism in the TNF-α promoter.

Tumour necrosis factor-α is a pro-inflammatory cytokine believed to play an important role in the pathogenesis of many inflammatory and severe infectious diseases. An example of a disease whose severity is known to be associated with TNF-α is fatal cerebral malaria which occurs in a small proportion of individuals infected with the malaria parasite *Plasmodium falciparum*. Clinical studies of cerebral malaria patients have demonstrated an association between host TNF-α levels and disease severity (Grau, G. E. *et al*. (1989) *New Engl. J. Med.* 320: 1586-1591; Kwiatkowski, D. *et al* (1990) *Lancet* 336: 1201-1204; Kern, P. *et al*. (1989) *Am. J. Med.* 57: 139-143) and experimental studies have revealed several ways in which excessive TNF-α production could promote cerebral malaria (Clark, I. A. (1987) *Parasitol. Today* 3: 300-305; Grau, G. E. *et al*. (1987) Science 237: 1210-1212).

In humans the gene for TNF-α resides within the class III region of the major histocompatibility complex (MHC) and several studies have shown that

individual variation in the level of TNF-α production can be linked to HLA type and to other polymorphic markers in the MHC class III region (Molving, J. *et al.* (1988) *Scand. J. Immunol.* 27: 705-716; Jacob, C.O. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87: 1233-1237; Pociot, F. *et al.* (1993) *Eur. J. Immunol.* 23: 224-231). These observations suggest that TNF-α responsiveness may be controlled by variable genetic elements in the MHC region but the precise location of these elements has yet to be identified.

In the search for genetic elements involved in the regulation of TNF-α gene expression there has been much interest in a group of single nucleotide polymorphisms located at position -238, -308 and -376 relative to the TNF-α transcriptional start site. All three polymorphisms are substitutions of adenine for guanine, the allelic types being hereinafter denoted $TNF_{-238G/-238A}$, $TNF_{-308G/-308A}$ and $TNF_{-376G/-376A}$ respectively.

Population studies have shown that these polymorphisms in the TNF-α promoter may be associated with susceptibility to disease. For example, a study carried out in a population of Gambian children provided evidence that homozygotes for the TNF_{-308A} allele have an increased risk of developing cerebral malaria (McGuire, W. *et al.* (1994) *Nature* 371: 508-511). The $TNF\text{-}_{-308A}$ allele has also been associated with susceptibility to a variety of other infectious diseases and chronic inflammatory diseases (Cabrera, M. *et al.* (1995) *J. Exp. Med.* 182: 1259-1264; Conway, D.J. *et al.* (1997) *Infec. Immun.* 65: 1003-1006; Nadel, S. *et al.* (1996) *J. Infec. Dis.* 174: 878-880; Roy, S. *et al.* (1997) *J. Infec. Dis.* 176: 530-532; Wilson, A. G. *et al.* (1994) *Eur. J. Immunol.* 24: 191-195; Cox, A. *et al.* (1994) *Diabetologia* 37: 500-503 and Louis, E. *et al.* (1996) *Gut* 39: 705-710). However, at this time

it remains an open question as to whether the disease associations with polymorphisms in the TNF-α promoter region are due to a direct effect on the TNF-α gene regulation, as opposed to linkage disequilibrium with functional polymorphisms elsewhere in the TNF-α locus or neighbouring genes.

The present inventors have surprisingly found that one of the known polymorphisms in the TNF-α promoter region is located within a site of DNA/protein recognition and thus has a direct functional effect on TNF-α transcription via altered transcription factor recruitment. The present inventors have further identified two novel DNA binding proteins which specifically recognise DNA sites in the region of the TNF-α promoter surrounding this polymorphism.

Accordingly, in a first aspect the invention provides a sequence-specific DNA binding protein having a molecular weight of 21kDa +/- 5kDa (hereinafter referred to as the 21kDa protein) which is capable of binding specifically to the sequence of nucleotides set forth in Figure 1a or 1b.

In a second aspect the invention provides a sequence-specific DNA binding protein having a molecular weight of 30kDa +/- 5kDa (hereinafter referred to as the 30kDa protein) which is capable of binding specifically to the sequence of nucleotides set forth in Figure 2.

The invention also provides isolated nucleic acids having the nucleotide sequences set out in Figure 1a or 1b or fragments thereof which are capable of specifically binding the above-described 21kDa DNA binding protein.

The invention also provides an isolated nucleic acid having the nucleotide sequence set out in Figure

2 or a fragment thereof which is capable of binding specifically to the above-described 30kDa DNA binding protein.

The proteins of the invention are DNA binding proteins which have been characterised as binding specifically to DNA sequences within the promoter region of the TNF-α gene. As will be described in Example 2 , the binding sites for these proteins were identified on the basis of solid phase DNAase I footprinting analysis of the region of the TNF-α promoter spanning positions -682 to -183 relative to the transcription start site using nuclear extracts from the cell line MonoMac6 which phenotypically resembles a well-differentiated human monocyte (Ziegler-Heitbrock, H. W.L. *et al* (1988) *Intl. J. Cancer* 41: 456-461). The precise position of the protein binding sites has been further elucidated using electrophoretic mobility shift assays (EMSA) as will be described in detail in Example 3 below. Thus, the first novel 21kDa DNA binding protein has been shown to bind to a site in the region of the TNF-α promoter extending from position -391 to position -374 relative to the transcription start site, having the nucleotide sequence set forth in Figure 1a. This region, which includes the previously identified -376 G/A polymorphism, is hereinafter referred to as the α site. The second novel 30kDa DNA binding protein has been shown to bind to a site in the region of the TNF-α promoter extending from position -365 to position -352 relative to the transcription start site, having the nucleotide sequence set forth in Figure 2. This region is hereinafter referred to as the β site. Complexes of the proteins of the invention bound to synthetic oligonucleotides comprising the sequence of the α and β sites have been isolated using UV-

crosslinking experiments and the molecular weight of the proteins has been determined using SDS-PAGE, as will be described in detail in Example 4.

The sequence-specific DNA binding properties of the 21kDa and 30kDa proteins can be readily exploited to purify the proteins on the basis of their binding specificity using techniques already known to those skilled in the art of protein purification. For example, the proteins may be purified from crude cell extracts (such as the nuclear extract of MonoMac6 cells described herein) on the basis of binding affinity by passing the nuclear extract over a solid support, such as a column matrix, to which have been attached synthetic oligonucleotides comprising a sequence corresponding to site α or site β. The desired proteins, bound to the matrix, may then be released by using appropriate buffers, known in the art, and further purified by biochemical procedures such as, for example, SDS-PAGE electrophoresis or Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC).

Partial aminoacid sequence of the purified proteins may be determined using methods known to those skilled in the art of protein sequencing. For example, the purified molecule may be subjected to an Edman degradation reaction or, alternatively, to tandem mass-spectrometry. Prior to undertaking these procedures, it may be advantageous to cleave the purified molecule into smaller peptide fragments, for example, by using a protein cleaving enzyme such as trypsin followed by separation of the different fragments by Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC).

The two regions of the TNF-α promoter denoted

herein site α and site β have been identified as protein binding sites on the basis of DNAase I footprinting and EMSA experiments and are therefore putative *cis*-acting regulatory elements within the TNF-α promoter. Of the three previously described polymorphisms in the TNF-α promoter only one, the -376 G/A polymorphism, occurs within one of the putative *cis*-acting regulatory elements. As will be described in detail in Example 5, the present inventors were able to demonstrate that the -376 G/A substitution has a direct effect on TNF-α transcription by placing 1.2kB of the human TNF-α promoter sequence upstream of a luciferase reporter gene and transiently expressing the construct in MonoMac6 cells. The -376 G/A polymorphism was introduced into the reporter gene construct by site-directed mutagenesis. Constructs containing the A substitution (-376A) showed a 35% increase in basal reporter gene expression compared to the wild-type promoter (376G).

The present inventors have further demonstrated that the pattern of protein binding to site α is markedly altered by the naturally occurring polymorphism at position -376. As will be described in detail in Examples 3 and 4, both EMA and UV-crosslinking experiments demonstrated that whilst the 21kDa DNA binding protein is able to bind to the α site of both the TNF_{-376A} and TNF_{-376G} alleles, in the presence of the TNF_{-376A} allele but not the TNF_{-376G} allele a second protein of molecular weight of 95kDa (hereinafter referred to as the 95kDa protein) can bind to the α site in addition to the 21kDa protein. Thus, sites α and β of the TNF-α promoter normally interact with the 21kDa and 30kDa proteins respectively and the TNF_{-376A} allele serves to recruit a third protein of about 95kDa to the α site. The

present inventors have further demonstrated with the use of EMSA supershift and competition experiments that the 95kDa protein is the ubiquitously expressed transcription factor Oct-1.

Knowledge of the specific DNA-protein binding interactions important in the regulation of TNF-α transcription can be used to design assays to identify compounds capable of disrupting the DNA-protein binding interactions and thus modulating TNF-α transcription. Compounds thus identified may be useful therapeutically in clinical situations in which it is desirable to up-regulate or down-regulate the level of TNF-α expression.
Accordingly, in a further aspect the invention provides a method of identifying compounds capable of modulating TNF-α gene expression, which method comprises the steps of:

(a) contacting an aqueous solution comprising the 21kDa DNA binding protein with a sample of the compound to form a reaction mixture;

(b) contacting the reaction mixture of part (a) with a DNA fragment comprising the sequence of nucleotides set forth in Figure 1a or Figure 1b;

(c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

In a further aspect the invention provides a method of identifying compounds capable of modulating TNF-α gene expression, which method comprises steps of:

(a) contacting an aqueous solution comprising the 21kDa DNA binding protein and the transcription factor protein Oct-1 with a sample of the compound to

form a reaction mixture;

(b) contacting the reaction mixture of part (a) with a DNA fragment comprising the sequence of nucleotides set forth in Figure 1b;

(c) observing the presence or absence of complexes comprising said DNA fragment.

In a further aspect the invention provides a method of identifying compounds capable of modulating TNF-α gene expression, which method comprises steps of:

(a) contacting an aqueous solution comprising the 30kDa DNA binding protein with a sample of the compound to form a reaction mixture;

(b) contacting the reaction mixture of part (a) with a DNA fragment comprising the sequence of nucleotides set forth in Figure 2.

(c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

The above methods of the invention can advantageously be performed in the form of an electrophoretic mobility shift assay (EMSA), as described in Example 3 below. A sample of the compound to be tested may be added to the reaction mixture prior to the addition of radiolabelled probe. Binding reactions with and without the test compound are then analysed by electrophoresis. Compounds capable of disrupting the specific DNA-protein binding interaction will result in an absence of the band corresponding to the complex in a similar manner to the competition experiments described in Example 3.

The invention also provides a method of

identifying compounds capable of modulating TNF-α gene expression, which method comprises steps of:

(a) contacting a DNA-protein complex comprising a DNA fragment comprising the sequence of nucleotides set forth in Figure 1a or 1b and the 21kDA DNA binding protein or the sequence of nucleotides set forth in Figure 2 and the 30kDa DNA binding protein or the sequence of nucleotides set forth in Figure 1b, the 21kDa DNA binding protein and the transcription factor protein Oct-1, with a sample of the compound;

(b) observing whether the DNA-protein complex is disrupted following contact with the compound.

In a further aspect the invention provides a method of identifying a compound capable of modulating TNF-α gene expression, which method comprises contacting a fragment of DNA comprising one or more of the sequences of nucleotides set forth in Figure 1a, Figure 1b or Figure 2 in the substantial absence of any other nucleotide sequences from the TNF-α promoter with a sample of the compound and detecting specific binding of the compound to the fragment of DNA.

The method of the invention is used to identify compounds capable of specifically binding to the α and β sites within the TNF-α promoter, which compounds may thus be useful as modulators of TNF-α transcription. Such compounds may be useful therapeutically in clinical situations in which it is desirable to up-regulate or down-regulate levels of TNF-α.

The readout of the assays of the invention may be provided by any technique known in the art to be useful in the detection of specific DNA/protein binding. Preferably, the oligonucleotides of interest are labelled, either with biochemical markers or radioisotopes and binding of compounds is screened by

techniques such as, for example, electrophoretic mobility shift assay (EMSA). Alternatively, specific binding interactions can be detected, without the need of labels, by proximity assay techniques such as, for example, flow cytometry or surface plasmon resonance. Advantageously, the assay method of the invention may be used to screen a combinatorial library of compounds in order to identify any compounds capable of specifically binding to site α or site β.

In a still further aspect the invention provides a reporter gene expression construct comprising:

a reporter gene encoding a transcriptional and/or translational product which can be directly or indirectly detected; and

a transcriptional control element comprising one or more of the sequences of nucleotides set forth in Figure 1a, Figure 1b or Figure 2 in the substantial absence of any other nucleotide sequence from a TNF-α promoter, transcription of the reporter gene being under the control of the transcriptional control element.

Reporter genes suitable for use in the expression construct include chloramphenicol acetyltransferase (CAT), β-galactosidase, firefly luciferase and green fluorescent protein. Following transfection into a host cell, reporter gene expression from the construct can be monitored either by direct measurement of mRNA or protein or by indirect measurement of properties of the protein such as enzymatic activity or fluorescence.

A suitable plasmid based reporter gene expression construct would include a reporter gene cDNA with a downstream polyadenylation signal, an upstream multiple cloning site into which the transcriptional

control element can be inserted, a synthetic polyadenylation signal upstream of the multiple cloning site to prevent read-through transcription from spurius promoter sequences in the vector backbone, a bacterial origin of replication and bacterial antibiotic resistance gene(s) to allow manipulation of the plasmid in a bacterial host strain. Suitable reporter gene expression vetors into which putative *cis*-acting regulatory elements of interest can be easily inserted are commercially available (for example from Promega). The expression construct may also contain *cis*-acting promoter elements from a heterologous RNA polymerase II dependent promoter which function to increase the basal level of reporter gene expression.

The invention further provides cells which have been transfected with the expression constructs of the invention. The invention still further provides a method of identifying compounds capable of modulating TNF-α gene expression, which method comprises comparing the amount of reporter gene expression in the cells of the invention in the presence of the compound with the amount of reporter gene expression in the absence of the compound or with the amount of reporter gene expression in cells transfected with a control reporter gene expression construct which does not contain the α and β sites of the TNF-α promoter, whereby compounds capable of modulating TNF-α gene expression are identified.

The method of the invention preferably uses cells which have been stably transfected to provide a cell line in which the reporter gene construct is stably integrated at a chromosomal location. To facilitate the production of stable cell lines one of the known

eukaryotic selectable markers may be added to the reporter gene expression construct. Following transfection, stable lines may be selected and propagated by culturing in media containing the appropriate selective agent.

In contrast to the assay methods described above, which detect specific binding of compounds to the α and β sites of the TNF-α promoter using physical techniques, the assay method, based upon the use of cells transfected with reporter gene constructs containing the α and β sites, uses a biological assay readout based on altered transcription. The assay may be used to identify compounds capable of modulating TNF-α expression which may be useful therapeutically in clinical situations in which it is desirable to up-regulate or down-regulate levels of TNF-α.

In a still further aspect the invention provides a method of screening human individuals for predisposition to inflammatory disease, which method comprises screening for the presence of a G to A transition polymorphism at position -376 in the promoter region of the TNF-α gene.

Having identified that the -376 G/A polymorphism has a direct functional effect on TNF-α transcription *in vitro*, the present inventors performed a study to address the question of whether the -376 G/A polymorphism also exerts a functional effect on TNF-α regulation *in vivo* by examining the association between the polymorphism and susceptibility to cerebral malaria in two distinct African populations. As previously mentioned, clinical studies on cerebral malaria patients have demonstrated an association

between host TNF-α levels and disease severity.

In the first part of this study the inventors analysed DNA samples from a large case control study of severe malaria in Gambian children. As will be described in detail in Example 6 the Gambian study identified TNF_{-376A} as an indipendent determinant of cerebral malaria with an estimated odds ratio of 4.3 when compared to a control group (95% confidence interval 1.5-12.8, P=0.008). On the basis of the Gambian study the inventors formulated the hypothesis that the TNF_{-376A} allele is a determinant of susceptibility to cerebral malaria. In order to test this hypothesis the inventors examined the frequency of the TNF_{-376A} allele in an indipendent case control study of severe malaria in in Kenyan children. In the Kenyan study population the TNF_{-376} allele was found to be in strong linkage disequilibrium with the TNF_{-238A} allele (Δ= 8.35 P< 0.001. Logistic regression analysis of the two linked polymorphisms, combined with ethnic group, indicated that possession of the TNF_{-376A} allele was associated with increased susceptibility to cerebral malaria (cerebral malaria cases vs controls: OR 5.0, 95% CI 1.5-17.0, P=0.010) while possession of the TNF_{-238A} allele was associated with protection (OR 0.2, 95% CI 0.08-0.7, P= 0.008). The present inventors have thus identified a strong relationship between the TNF_{-376A} allele and susceptibility to cerebral malaria in two populations with widely divergent genetic features living on opposite sites of the African continent.

Cerebral malaria is just one example of an inflammatory disease in which TNF-α is known to play an important role. Given that the -376 G/A polymorphism has been shown by the present inventors to have a direct functional effect on the basal level

of TNF-α transcription there is a reasonable expectation that the TNF_{-376A} allele is also associated with susceptibility to other inflammatory diseases in which TNF-α is known to be an important factor such as, for example, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus and the like.

Accordingly, the method of the invention may be used to screen for susceptibility to any inflammatory disease in which TNF-α is known to be important. The method of the invention is particularly useful in screening for susceptibility to cerebral malaria.

The method of the invention involves genotyping individuals to establish whether they carry TNF_{-376A} or TNF_{-376G} alleles and the genotyping may be carried out according to any of the techniques known to those skilled in the art of genetic screening. For example, genotyping may be accomplished with the method used by the present inventors in Example 6 part (b), namely PCR ELISA, on samples of genomic DNA using differential hybridisation with allele specific oligonucleotide probes. The following oligonucleotide sequences are preferred for use as allele specific probes:

5'-CTGTCTGGAAgTTAGAAGGA (TNF_{-376G} allele)
5'-CTGTCTGGAAaTTAGAAGGA (TNF_{-376A} allele)

The present invention may be further understood with reference to the following Examples together with the accompanying Figures in which:

Figure 1 shows the nucleotide sequence of the TNF-α promoter from position -391 to position -374 relative to the transcription start site, corresponding to the α site; 1a is the wild type TNF_{-376G} allele, 1b is the

polymorphic variant TNF_{-376A} allele.

Figure 2 shows the nucleotide sequence of the TNF-α promoter from position -365 to position -352 relative to the transcription start site, corresponding to the β site.

Figure 3 shows the results of a solid phase DNAase I footprinting experiment using a radiolabelled probe spanning the region from position -682 to position -183 of the TNF-α promoter, in the absence of nuclear extract (lanes 2 and 5) or incubated with nuclear extracts prepared from unstimulated MonoMac6 cells (lane 3) or MonoMac6 cells stimulated with lipopolysaccharide (LPS) (lane 4). Lane 1 is the Maxam Gilbert sequencing ladder for the same region of the TNF-α promoter.

Figure 4 shows the probes used in an electrophoretic mobility shift assay (EMSA) investigating the precise localisation of the α site in the TNF-α promoter region. α_G and α_A probes correspond to the nucleotide sequence extending from position -407 to position -373 of the TNF-α promoter, with either a G or an A at position -376. Proximal α_G and α_A probes correspond to the nucleotide sequence extending from position -391 to position -373 of the TNF-α promoter, with either a G or an A at position -376. Distal α probe corresponds to the sequence extending from position -407 to position -386 of the TNF-α promoter.

Figure 5 shows the results of an electrophoretic mobility shift assay (EMSA) investigating the precise localisation of the α site in the TNF-α promoter region. Nuclear extracts from unstimulated MonoMac6

cells were incubated with a radiolabelled α_G (lane 1), α_A (lane 4), distal α (lane 2), proximal α_G (lane 3) or proximal α_A (lane 5) probe.

Figure 6 shows an electrophoretic mobility shift assay (EMSA) investigating the binding of Oct-1 to the α site in the presence of an A substitution at position -376. Nuclear extracts from unstimulated MonoMac6 cells were incubated with a radiolabelled α_G (lanes 1 to 7) or α_A (lanes 8 to 14) probe either alone (lanes 1 and 8) or in the presence of anti-Oct1 antibody (lanes 2 and 9), anti-p50 antibody (lanes 3 and 10) or unlabelled competitor probes (lanes 4 to 8 and 11 to 14).

Figure 7 shows the probes used in an electrophoretic mobility shift assay (EMSA) investigating the precise localisation of the β site in the TNF-α promoter region. β corresponds to the sequence from nucleotide -372 to nucleotide -352 of the TNF-α promoter; β-1 corresponds to the sequence from nucleotide -365 to nucleotide -345 of the TNF-α promoter; β-2 corresponds to the sequence from nucleotide -359 to nucleotide -339 of the TNF-α promoter.

Figure 8 shows the results of electrophoretic mobility shift assay (EMSA) investigating the precise localisation of the β site in the TNF-α promoter region. Nuclear extracts from LPS stimulated (lane 3) or unstimulated (lane 2 and 4-14) MonoMac6 cell were incubated with a radiolabelled β (lane 2, 3 and 11), β-1 (lane 13) or β-2 (lane 14) probe. A competition assay was also performed with a radiolabelled β probe in the presence of a 10x molar excess of an unlabelled β (lane 4), β-1 (lane 8), β-2 (lane 10) or EGR site

(F:agctAAATCCCCGCCCCCGCGATGGA) (lane 6) probe or in the presence of a 100x molar excess of unlabelled β (lane 5), β-1 (lane 9), β-2 (lane 11) or EGR site (lane 7) probe.

Figure 9 shows the results of UV-crosslinking experiments investigating proteins binding at the α and β sites of the TNF-α promoter. EMSA binding reactions were performed with radiolabelled double stranded oligonucleotide probes comprising the α site (with G or A at position -376, for complex I and II respectively) or the β site (complex III), in which five dT nucleotides were substituted with BrdU (as shown in Figure 10 below), complexes were crosslinked by UV light, excised and resolved using a 4-12% gradient polyacrilamide gel, either (a) directly or (b) after immunoprecipitation with anti-Oct1 or control antibody.

Figure 10 shows the nucleotide sequence of the probes used in the UV-crosslinking experiment described in Example 4. 5BrdU is shown as a Q.

<u>Example 1</u> <u>Preparation of Nuclear Extracts.</u>

MonoMac6 cells (10-20x10^6) maintained in culture as previously described (Ziegler-Heitbrock, H. W. L. et al. *Int. J. Cancer*. 41: 456-461 (1988)) were either harvested unstimulated or stimulated with 100ng/ml lipopolysaccharide (LPS) for 1 hour and then harvested. Nuclear extracts were prepared according to the method of Schreiber, E. et al. *Nucleic Acids Res.* 17: 6419 (1989).

Example 2 Solid Phase DNAase I Footprinting.
Experimental method

Radiolabelled probes spanning the TNF-α promoter region from position -682 to position -183 relative to the transcription start site were generated by PCR using antisense primer 5'-GTTGGGGACACACAAGCATC (end labelled with γ^{32}P-dATP using T4 polynucleotide kinase) and biotinylated primer 5'-GCATTATGAGTCTCCGGGTC using TNFwt(-1173)-pXP1 plasmid DNA as template (construction of this plasmid is described in Example 5 part (A) below. The DNAase I footprinting procedure itself was carried out according to the technique described by Sandaltzopoulos and Becker (*Nucleic Acids Research*. 22: 1511-1515 (1994)). Briefly, the DNA-binding reactions comprised radiolabelled DNA probe (40,000 cpm) absorbed onto magnetic Dynabeads M-280-Streptavidin in binding reaction buffer (12mM HEPES, pH 7.8, 80-100mM Kcl, 1mM EDTA, 12% glycerol and poly (dI-dC) either alone (naked DNA) or incubated with 20μg crude nuclear extract prepared from MonoMac6 cells according to Example 1. The products of the DNA-binding reactions were subjected to DNAase I digestion 0.12-0.25U for 30 seconds, washed and analysed on a 7% acrylamide 7M urea gel. Areas of protection were localised by comparison with a Maxam-Gilbert sequencing ladder.

Results

Figure 3 shows that two novel sites of DNA-protein interaction are seen with nuclear extracts from LPS stimulated and unstimulated MonoMac6 cells. The first, designated α, is located between position -404 and -374 of the TNF-α promoter region. The second,

designated β, is located between position -371 and -352. A further region of protection at position -600 is seen with nuclear extracts from LPS stimulated cells only and corresponds to the previously known NF-κB site cluster.

Example 3 Electrophoretic Mobility Shift Assay. Experimental method

Double stranded oligonucleotide probes were annealed and radiolabelled with an equimolar amount of α^{32}P-dCTP using DNA polymerase I. Binding reactions were assembled containing 1μg crude nuclear extract prepared according to Example 1 and 4000 cpm radiolabelled probe in 10mH HEPES (pH 7.8), 50mM Kcl, 1mM EDTA, 1mM EGTA, 12.5% glycerol, 1μg polyIdC and incubated for 20 minutes at room temperature. The binding reactions were analysed by electrophoresis in a non-denaturing 5% polyacrylamide gel at 4°C in 0.5% TBE buffer. EMSA supershift analysis was performed by pre-incubation of the reaction mixture with an appropriate antiserum (Santa Cruz) at room temperature for 10 minutes prior to addition of the radiolabelled probe.

Results

a) Precise localisation of α site

In order to identify the precise region of the TNF-α promoter corresponding to the α site, and EMSA assay was carried out as described above, incubating the different probes shown in Figure 4 with nuclear extracts from unstimulated MonoMac cells. Figure 5 shows that a low molecular weight complex (complex I) was observed with the α_G (lane 1) and α_A (lane 4)

probes, as well as with the proximal α_G (lane 3) and α_A (lane 5) probes. An additional high molecular weight complex (complex II) was observed only with the α_A(lane 4) and proximal α_A (lane 5) probes, indicating that the G/A polymorphism at position -376 has a direct effect on recruitment of DNA-binding proteins at the α site. No complex was observed with the distal α probe (lane 2), showing that the 5' region of α_G or α_A is not involved in any DNA-protein interaction. These data, combined with those obtained from the Footprinting experiment of Example 2, suggest that the α site is localised between nucleotide -391 and nucleotide -374 of the TNF-α promoter.

b) <u>Characterisation of complex II</u>

The nature of complex II was further investigated by an EMSA supershift and competition assay, the results of which are shown in Figure 6. Complex II but not complex I was neutralised by the presence of antibody to Oct-1 (lanes 2 and 9) whilst neither complex was affected by anti-p50 (lanes 3 and 10). Complex I was completed by 100x molar excess of probe α_G (lane 4) or probe α_A (data not shown), both shown in Figure 4, but not by 100x molar excess of an oligoduplex probe matching an Oct-1 binding site from the human histone 2b gene (F:agctTCGCTTATGCAAATAAGGTGA) (lanes 5 and 6) or a probe matching the EGR site (F:agctAAATCCCCGCCCCCGCGATGGA) (lane 7). In contrast, complex II was completed by a molar excess of α_A (lane 11) and the probe matching the Oct-1 binding site (lanes 12 and 13) but not by the probe matching the EGR site (lane 14 or probe α_G (data not shown). The EMSA supershift and competition data indicate that in the presence of TNF_{-376A} allele but not in the presence

of the TNF_{-376G} allele Oct-1 binds to the α site in addition to the 21KDa protein to form complex II.

c) Precise localisation of β site

In order to identify the precise region of the TNF-α promoter corresponding to the β site, an EMSA assay was carried out as described above, utilising the probes shown in Figure 7.
Figure 8 shows that formation of a complex (complex III) was observed with the β (lanes 2,3 and 12) and β-1 (lane 13) probes but not with the β-2 probe (lane 14). Furthermore, formation of complex III with the β probe was completed by a molar excess of the β (lanes 4 and 5) or the β-1 (lanes 8 and 9) probe but not by the β-2 probe (lanes 10 and 11) or by an irrelevant probe (lane 6 and 7).
These results, combined with those obtained from the Footprinting experiment of Example 2, suggest that the β site is localised between nucleotide -365 and -352 of the TNF-α promoter region.

Example 4 UV-crosslinking

Experimental methods

UV-crosslinking was carried out by first performing EMSA experiments using radiolabelled oligoduplex probes in which specified dT nucleotides were substituted with BrdU, as shown in Figure 10. The EMSA gel was then illuminated with UV radiation at 302nm for 30 minutes at 4°C and exposed to autoradiography film for four hours at the same temperature. The autoradiography film was used to locate regions of the EMSA gel corresponding to the specific protein-DNA complexes. The relevant fragments of gel were then

excised and the complexes eluted in 2x SDS buffer (100mM Tris-Cl pH 6.8, 200mM DTT, 2% SDS, 20% glycerol) at 37°C overnight. The complexes thus isolated were then resolved using a 4-12% gradient polyacrilamide gel either directly or after immunoprecipitation as previously described (Hansen, S.K. et al. *EMBO J.* 11: 205-213).

Results

Figure 9 shows the results of UV-crosslinking experiments to investigate proteins binding at site α and β of the TNF-α promoter using α and β site oligoduplex probes. Figure 9(a) 4-12% gradient SDS-PAGE of complexes formed by incubation of crude nuclear extracts from unstimulated MonoMac6 cells with α site probes containing A (lane 1) or G (lane 2) at position -376 or with β site probes (lane 3), all shown in Figure 10. The molecular weight of the complexes were determined by comparing their electrophoretic mobility with that of molecular weight markers. The molecular weight of the proteins binding to site α or β were then calculated by subtracting the molecular weight of the oligonucleotide probes. Two proteins of about 21kDa and 30kDa were shown to bind the α and β sites, respectively. Furthermore, another protein of about 95kDa was shown to bind, together with the 21kDa protein, only to the α_A site but not to the α_G site. These estimates are accurate to +/- 5kDa, since the migration of the complexes may be influenced by various structural variables. Figure 9(b) shows SDS-PAGE after immunoprecipitation of complexes I and II with anti-Oct-1 or a control unrelated antibody (anti-p50 NF-κB). UV crosslinked forms of complex II but not of complex I could be specifically

immunoprecipitated by anti-Oct-1 antibodies, showing that the protein of about 95kDa is Oct-1. Both figure 9(a) and 9(b) show that the binding of Oct-1 is specific for the TNF_{-376A} allele.

Example 5 Reporter gene analysis

(a) Plasmid construction

The plasmid TNFwt (-1173)-pXP1 was constructed by placing a ~1.2kb fragment of the wild type TNF-α promoter and 5' untranslated region from position -1173 to position +130 relative to the transcriptional start site upstream of a firefly luciferase reporter gene in the eukaryotic expression vector pXP1 (described by Nordeen S.K. (1988) *Biotechniques* 6: 454-456). The -1173 to +130 fragment of wild type TNF-α was obtained as a HindIII/NdeI fragment from the previously described plasmid -1173-CAT (Udalova I. A. *et al.* (1995) *Dorklady Academii Nauk.* 342: 413-417). The corresponding fragment containing the G to A substitution at position -376 was synthetised by site directed mutagenesis in the construct -1173-CAT, using previously published methodology (Stuber F. *et al* (1996) *J. Inflamm.* **46**: 42-50).(1996)).

(b)Transient transfection of MonoMac6 cells

Transfection of MonoMac6 cells was carried out using the modified DEAE-dexran method (Shakhov, A et al. *J.Exp. Med.* 171: 35-47 (1990)) using 100μg/ml DEAE dextran and 2μg plasmid DNA. The cells were allowed to recover for 24 hours post-transfection and then either left unstimulated or stimulated with 200ng/ml LPS. The cells were harvested after 40 hours with cell lysate volumes adjusted to equalise total cellular protein content between samples. Firefly luciferase activity

was measured using a manual Turner TD20e luminometer (Promega).

Results

The results of transisent transfection experiments using MonoMac6 cells are summarised in Table 1 below:

Table 1

	No Insert	376G	376A	*P* Value*
Basal	0.23±0.07	16.1±1.7	21.7±2.4	0.002
LPS-stimulated	0.25±0.1	189.9±19.3	200.3±13.7	*ns*
Ratio of LPS stimulated: basal	1.1±0.5	12.3±1.4	10.1±1.5	0.007

**P* values shown for comparison of 376G and 376A constructs by two-tailed paired t-test. MonoMac6 cells were transfected with pXP1 vector containing no insert, which served as a negative control, or with one of two allelic forms of the TNF-α promoter region which differed by a single nucleotide substitution at position -376, either G (denoted as 376G) or A (denoted as 376A). The mean of 7 independent transient transfection experiments is shown (± SEM) using two independent plasmid preparations. Cells were unstimulated to determine basal expression or stimulated with a high dose of LPS (200ng/ml). Luciferase activity of cell lysates was determined using a luminometer (with results corrected for total

cell protein) and is expressed as luminometer units per mg protein.

Example 6 Clinical case-control studies.

(A) Study design

Children under 10 years of age were recruited to two independent case-control studies in the Gambia and Kenya. The Gambian study was carried out near Banjul and was hospital based as described previously (Hill, A.V.S. et al. *Nature*. 352: 595-600 (1991)). In the Kenyan study cases of severe malaria recurited at the Kilifi District Hospital were matched for age with community controls (Newbold, C. et al. *J. Trop. Med. Hygiene*. 57: 398-398 (1997); Snow, R.W. et al. *Trans. R. Soc. Trop. Med. Hyg*. 87: 386-390 (1993)). The studies were approved by the MRC/Gambian joint ethical committee and the KEMRI ethical committee. Cerebral malaria was defined as a Blantyre coma score of 2 or less (Molyneux, M.E. et al. *Q.J. Med*. 71: 441-459 (1989)) persisting for more than 30 minutes after any convulsions had ceased in a child with *P.falciparum* parasites on thick blood film and no evidence of meningitis or any other cause of coma. The Gambian control group comprised children seen in clinic with a variety of mild non-malarial illnesses that did not require admission to hospital. The Kenyan control group comprised healthy age-matched children from the same community as the index cases, recruited irrespective of presence or absence of malarial parasitaemia. The mean age of children in the Gambian study was 3.9 years for cerebral malaria cases and 2.9 years for the controls, whilst in the Kenyan study it was 2.7 years and 2.5 years respectively. Data was also analysed from Gambian children with severe

malarial anaemia (haemoglobin <5g/dl) or mild malaria (defined as an uncomplicated febrile illness in a child with asexual *P. falciparum* parasites on the blood film without other satisfactory explanation for the fever).

(B) Genotyping of TNF_{376} and TNF_{-238}.

Genotyping was carried out on randomised samples of genomic DNA, blind to disease outcome, by PCR ELISA (Boehringer Mannheim) using differential hybridisation with sequence specific probes. Nested PCR amplification generated a digoxigen labelled 375 nucleotide product spanning position -558 to position -183 of the TNF-α promoter using 100ng of genomic DNA as a template. This product was alkaline denatured and hybridised in streptavidin coated microtitre plates with allele-specific 5' biotinylated oligonucleotide probes at 55°C for 2 hours. Allele-specific capture probes used for TNF_{-238} were 5'-CCTCGGAATCgGAGCAGGGA and 5'-CCTCGGAATCaGAGCAGGGA at 2.5 pmol/ml; for TNF_{-376} 5'-CTGTCTGGAAgTTAGAAGGA and 5'-CTGTCTGGAAaTTAGAAGGA at 15 pmol/ml. A split plate design was used for each polymorphism, including positive and negative controls. Hybridised DNA was labelled by incubation with 2mU anti-digoxigenin peroxidase conjugate for 30 munutes at 37°C. After addition of 0.2mg of ABTS substrate plates were read on at ELISA plate reader at 405nm (reference filter 492nm). The accuracy of genotyping data obtained by PCR ELISA was confirmed by DNA sequencing.

Results

Results from the Gambian case control study are

summarised in Table 2 below:

Table 2

	Cerebral malaria	Severe malaria anaemia	Mild malaria	Non-malaria controls
TNF_{-376A}				
n	384	132	349	371
Heterozygotes	24(6.3%)	5 (3.8%)	13(3.7%)	9(2.4%)
Homozygotes	1(0.3%)	0	1(0.3%)	1(0.3%)

n number of individuals in each clinical category for the Gambian case-control study. In a 2x2 analysis possession of the TNF_{-376A} allele was associated with an odds ratio of 2.5 (95% CI 1.2-5.3) x^2=6.21 *P*=0.013) for cerebral malaria versus non-malaria controls.

CLAIMS

1. A sequence-specific DNA binding protein having a molecular weight of 21kDa +/- 5kDa which is capable of binding specifically to a DNA molecule having the sequence of nucleotides set forth in Figure 1a or 1b.

2. A sequence-specific DNA binding protein having a molecular weight of 30kDa +/- 5kDa which is capable of binding specifically to a DNA molecule having the sequence of nucleotides set forth in Figure 2.

3. An isolated nucleic acid having the sequence of nucleotides set forth in Figure 1a.

4. An isolated nucleic acid having the sequence of nucleotides set forth in Figure 1b.

5. An isolated nucleic acid as claimed in claim 3 or claim 4 or a fragment thereof which is capable of specifically binding to the DNA binding protein of claim 1.

6. An isolated nucleic acid having the sequence of nucleotides set forth in Figure 2.

7. An isolated nucleic acid as claimed in claim 6 or a fragment thereof which is capable of specifically binding to the DNA binding protein of claim 2.

8. A reporter gene expression construct comprising:

a reporter gene encoding a transcriptional and/or translational product which can be directly or indirectly detected; and

a transcriptional control element comprising one

or more of the sequences of nucleotides set forth in Figure 1a, Figure 1b or Figure 2 in the substantial absence of any other nucleotide sequence from a TNF-α promoter, transcription of the reporter gene being under the control of the transcriptional control element.

9. A reporter gene expression construct as claimed in claim 8 which further comprises one or more *cis*-acting promoter or enhancer elements from a heterologous promoter.

10. Cells transfected with the reporter gene construct claimed in claim 8 or claim 9.

11. A method of identifying a compound capable of modulating TNF-α gene expression, which method comprises contacting a fragment of DNA comprising one or more of the sequences of nucleotides set forth in Figure 1a, Figure 1b or Figure 2 in the substantial absence of any other nucleotide sequences from a TNF-α promoter with a sample of the compound and detecting specific binding of the compound to the fragment of DNA.

12. A method of identifying compounds capable of modulating TNF-α gene expression, which method comprises:

comparing the difference in the amount of reporter gene expression in the cells of claim 10 in the presence of the compound with the amount of reporter gene expression in the absence of the compound or with the amount of reporter gene expression in cells transfected with a control reporter gene expression construct which does not

contain one or more of the sequences of nucleotides set forth in Figure 1a, Figure 1b or Figure 2, whereby compounds capable of modulating TNF-α gene expression are identified.

13. A method of identifying compounds capable of modulating TNF-α gene expression, which method comprises steps of:
(a) contacting an aqueous solution comprising the DNA binding protein claimed in claim 1 with a sample of the compound to form a reaction mixture;
(b) contacting the reaction mixture of part (a) with a DNA fragment comprising the sequence of nucleotides set forth in Figure 1a or Figure 1b; and
(c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

14. A method of identifying compounds capable of modulating TNF-α gene expression, which method comprises steps of:
(a) contacting an aqueous solution comprising the DNA binding protein claimed in claim 1 and the transcription factor protein Oct-1 with a sample of the compound to form a reaction mixture;
(b) contacting the reaction mixture of part(a) with a DNA fragment comprising the sequence of nucleotides set forth in Figure 1b; and
(c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

15. A method of identifying compounds capable of modulating TNF-α gene expression, which method comprises steps of:

(a) contacting an aqueous solution comprising the DNA binding protein claimed in claim 2 with a sample of the compound to form a reaction mixture;
(b) contacting the reaction mixture of part(a) with a DNA fragment comprising the sequence of nucleotides set forth in Figure 2; and
(c) observing the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment.

16. A method as claimed in any one of claims 13 to 15 wherein said DNA fragment is radiolabelled and the presence or absence of complexes comprising said DNA binding protein bound to said DNA fragment is determined by electrophoretic mobility shift assay.

17. A method of identifying compounds capable of modulating TNF-α gene expression, which method comprises steps of:
(a) contacting a DNA-protein complex comprising a DNA fragment comprising the sequence of nucleotides set forth in Figure 1a or 1b and the DNA binding protein claimed in claim 1 or the sequence of nucleotides set forth in Figure 2 and the DNA binding protein claimed in claim 2 or the sequence of nucleotides set forth in Figure 1b, the DNA binding protein claimed in claim 1 and the transcription factor protein Oct-1, with a sample of the compound; and
(b) observing whether the DNA-protein complex is disrupted following contact with the compound.

18. A compound capable of modulating TNF-α gene expression, which compound has been identified using the method of any of claims 11 to 17.

19. A method of screening human individuals for predisposition to inflammatory disease, which method comprises screening for the presence of a G to A transition polymorphism at position -376 in the promoter region of the TNF-α gene.

20. A method as claimed in claim 19 wherein the inflammatory disease is cerebral malaria.

21. A method as claimed in claim 19 or claim 20 wherein screening for the presence of a G to A transition polymorphism at position -376 in the promoter region of the TNF-α gene is carried out using PCR ELISA.

22. A method as claimed in claim 21 wherein said PCR ELISA is carried out using allele-specific oligonucleotide probes having the following sequences:

5'-CTGTCTGGAAGTTAGAAGGA

5'-CTGTCTGGAAATTAGAAGGA

23. A sequence-specific DNA binding protein as claimed in claim 1 and substantially as described herein with reference to the accompanying Examples.

24. A sequence-specific DNA binding protein as claimed in claim 2 and substantially as described herein with reference to the accompanying Examples.

25. An isolated nucleic acid as claimed in claim 3 and substantially as described herein with reference to the accompanying Figures.

26. An isolated nucleic acid as claimed in claim 5 and substantially as described herein with reference

to the accompanying Figures.

27. An isolated nucleic acid as claimed in claim 7 and substantially as described herein with reference to the accompanying Figures.

28. A method of screening human individuals for predisposition to inflammatory disease as claimed in claim 19 and substantially as described herein with reference to the accompanying Examples.

FIG. 1a.

5'-GCATCCTGTCTGGAA<u>G</u>TT

FIG. 1b.

5'-GCATCCTGTCTGGAA<u>A</u>TT

FIG. 2.

5'-ACAGACCACAGACC

FIG. 3.

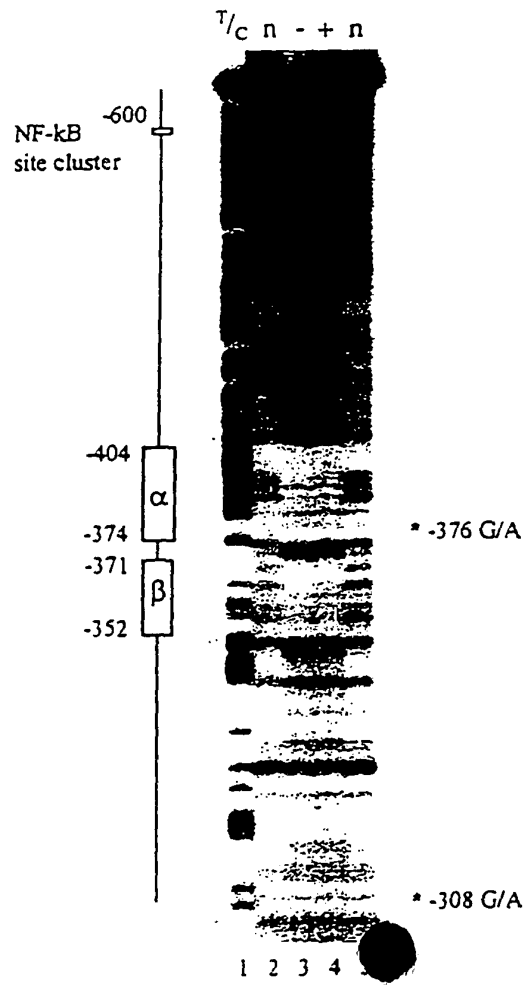

FIG. 4.

	404nt	374nt
	TTCTAGTTCTATCTTTTTCCTGCATCCTGTCTGGAAgTTAGAAGGAA	
Probe α_G	GTTCTATCTTTTTCCTGCATCCTGTCTGGAAgTTA	
Probe α_A	GTTCTATCTTTTTCCTGCATCCTGTCTGGAAaTTA	
Probe distal α	GTTCTATCTTTTTCCTGCATCC	
Probe proximal α_G	GCATCCTGTCTGGAAgTTA	
Probe proximal α_A	GCATCCTGTCTGGAAaTTA	

FIG. 5.

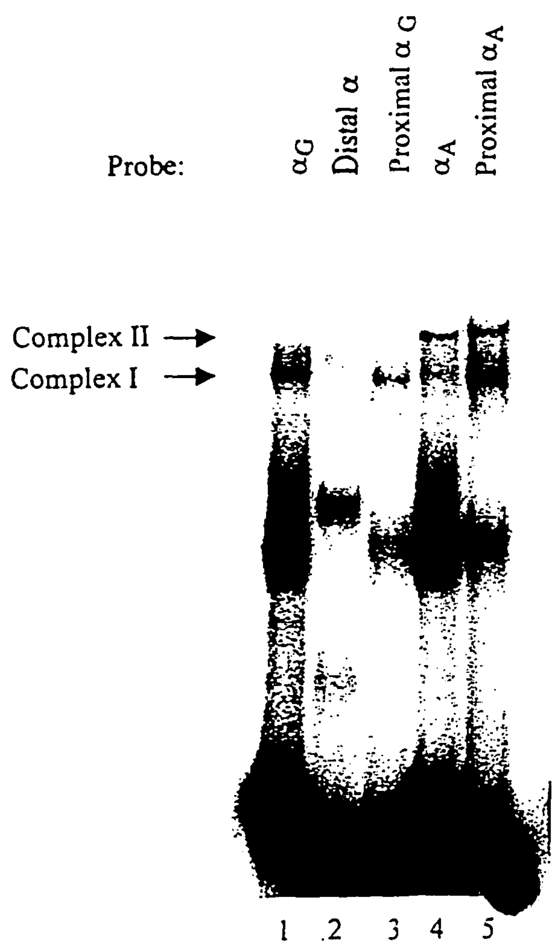

FIG. 6.

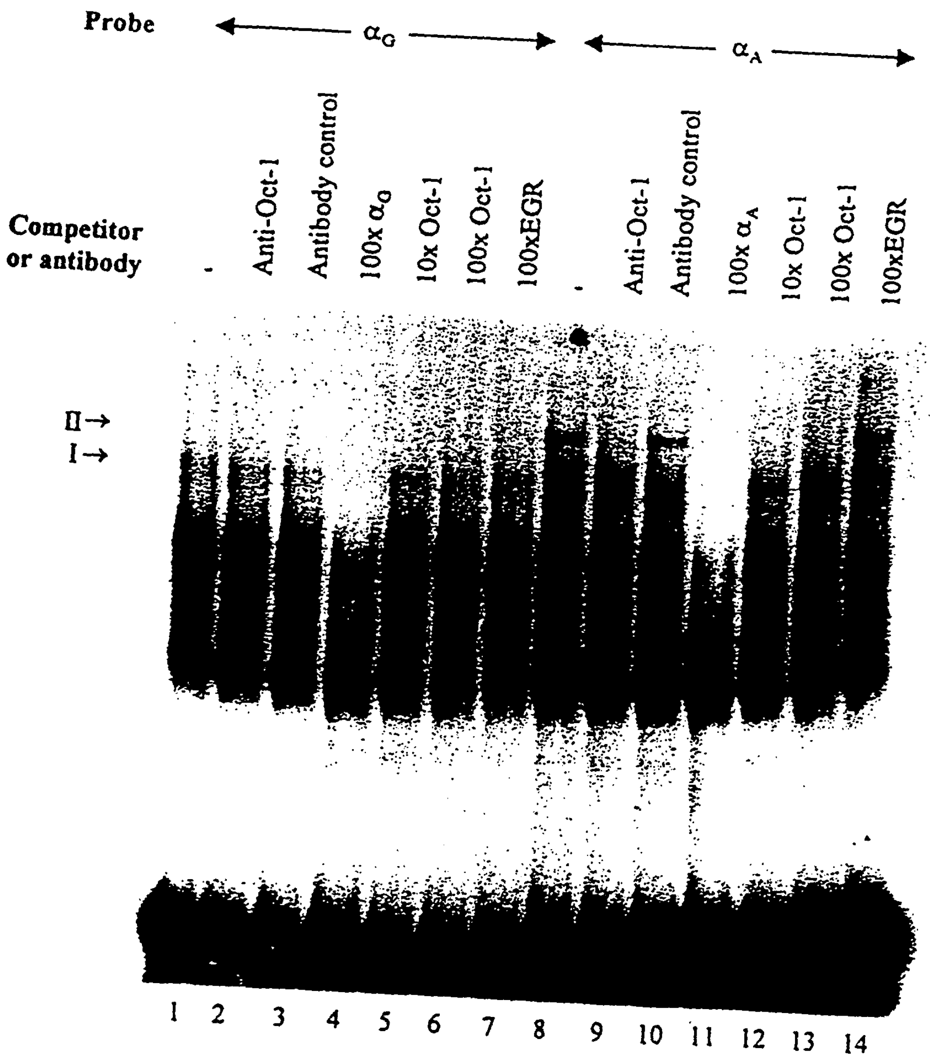
Probe
α_G
α_A
Competitor or antibody
-
Anti-Oct-1
Antibody control
100x α_G
10x Oct-1
100x Oct-1
100xEGR
-
Anti-Oct-1
Antibody control
100x α_A
10x Oct-1
100x Oct-1
100xEGR
II→
I→
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG. 7.

	-372nt	-352nt
	TAGAAGGAAACAGACCACAGACCTGGTCCCCAAAAG	
Probe β	TAGAAGGAAACAGACCACAGACCTG	
Probe β-1	ACAGACCACAGACCTGGTCCC	
Probe β-2	CACAGACCTGGTCCCCAAAAG	

FIG. 8.

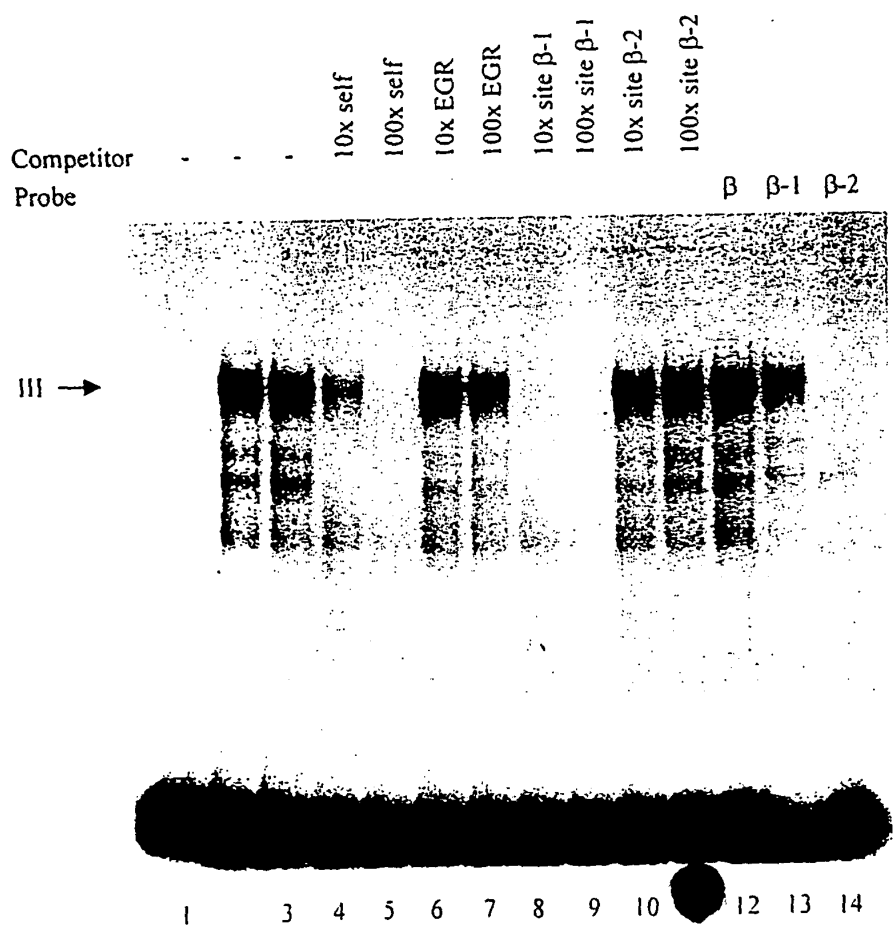

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FIG. 9a.

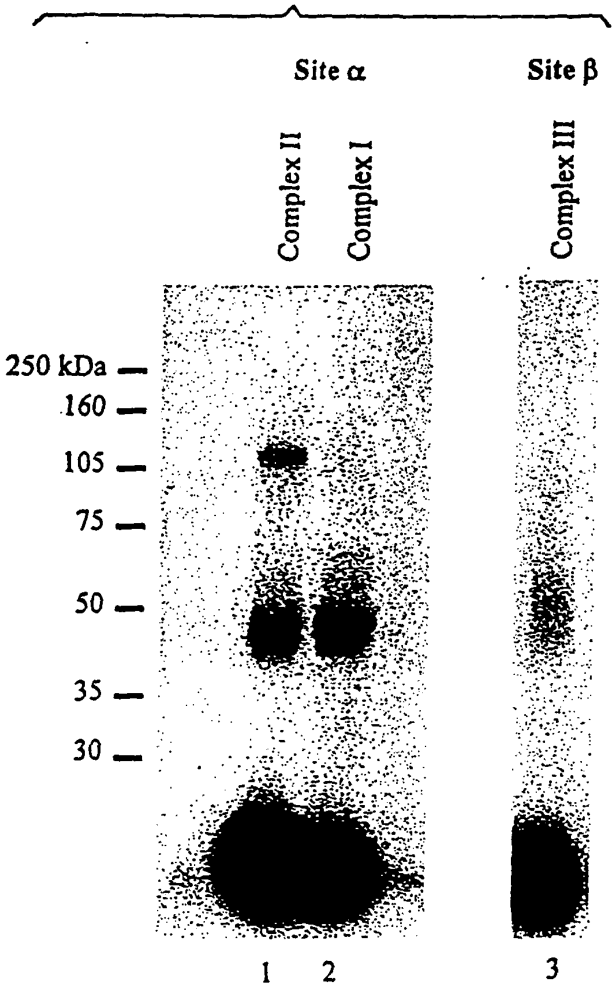

FIG. 9b.

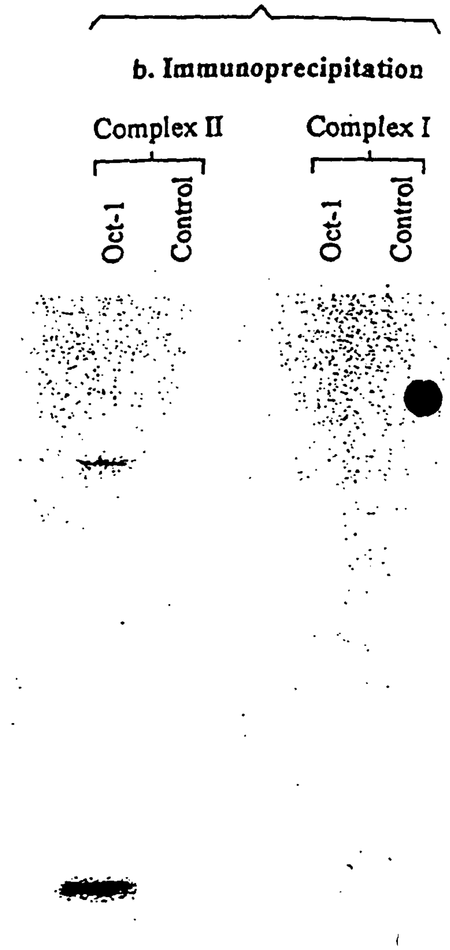

FIG. 10.

Site α_G	agctGTTCTATCTTTQTCCQGCATCCQGTCQGGAAGQTA agctQAACQQCCAGACAGGAQGCAGGAAAAAGAQAGAAC
Site α_A	agctGTTCTATCTTTQTCCQGCATCCQGTCQGGAAAQTA agctQAAQTQCCAGACAGGAQGCAGGAAAAAGAQAGAAC
Site β	agctQAGAAGGAAACAGACCACAGACCQG agctCAGGTCTGQGGQCTGQTQCCQT[illegible]A

FIG. 1a.

5'-GCATCCTGTCTGGAAGTT

FIG. 1b.

5'-GCATCCTGTCTGGAAATT

FIG. 2.

5'-ACAGACCACAGACC

FIG. 3.

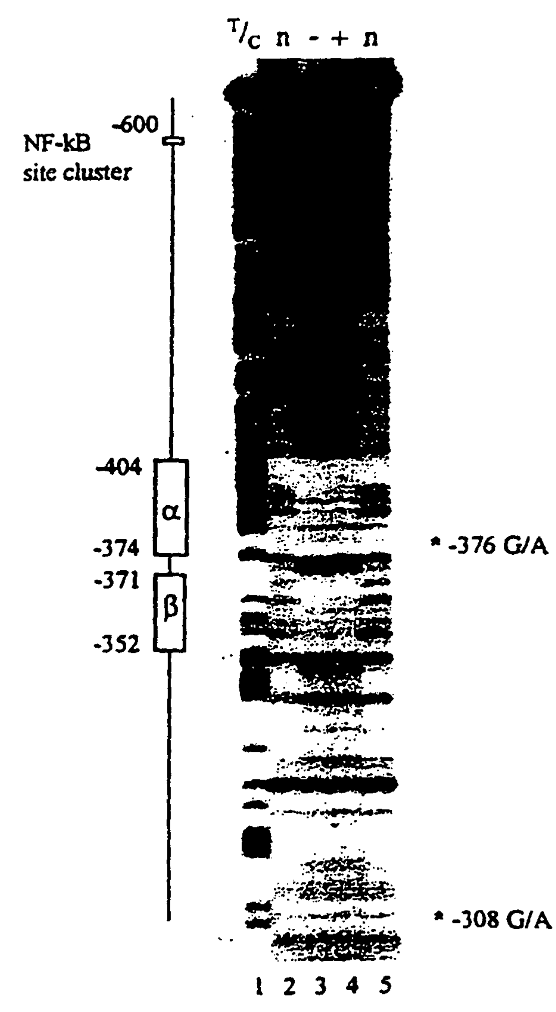

FIG. 4.

	404nt ... 374nt
	TTCTAGTTCTATCTTTTTCCTGCATCCTGTCTGGAAgTTAGAAGGAA
Probe α_G	GTTCTATCTTTTTCCTGCATCCTGTCTGGAAgTTA
Probe α_A	GTTCTATCTTTTTCCTGCATCCTGTCTGGAAaTTA
Probe distal α	GTTCTATCTTTTTCCTGCATCC
Probe proximal α_G	GCATCCTGTCTGGAAgTTA
Probe proximal α_A	GCATCCTGTCTGGAAaTTA

FIG. 5.

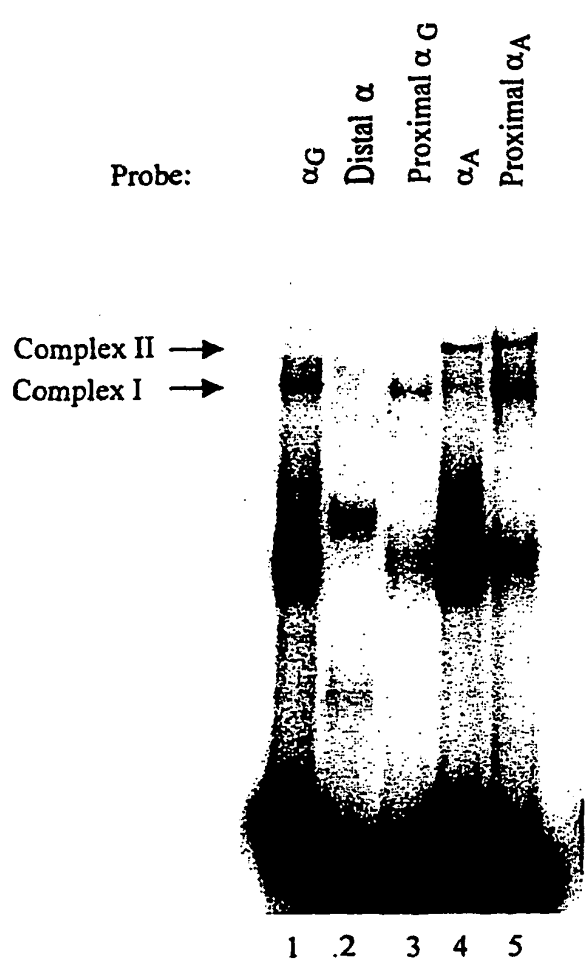

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FIG. 6.

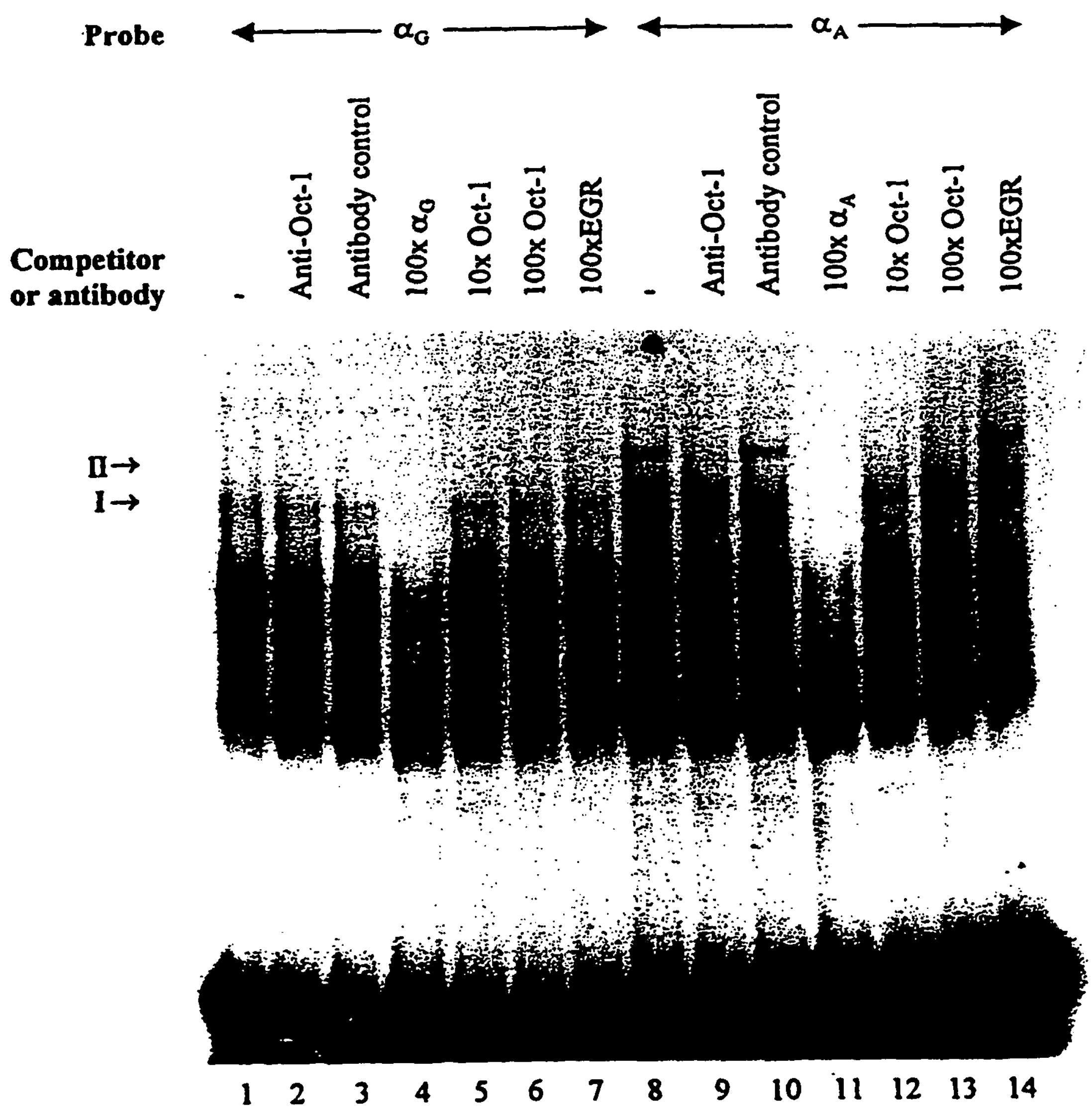

Probe
α_G
α_A
Competitor or antibody
-
Anti-Oct-1
Antibody control
100x α_G
10x Oct-1
100x Oct-1
100xEGR
-
Anti-Oct-1
Antibody control
100x α_A
10x Oct-1
100x Oct-1
100xEGR
II→
I→
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG. 7.

	-372nt	-352nt
	TAGAAGGAAACAGACCACAGACCTGGTCCCCAAAAG	
Probe β	TAGAAGGAAACAGACCACAGACCTG	
Probe β-1	ACAGACCACAGACCTGGTCCC	
Probe β-2	CACAGACCTGGTCCCCAAAAG	

FIG. 8.

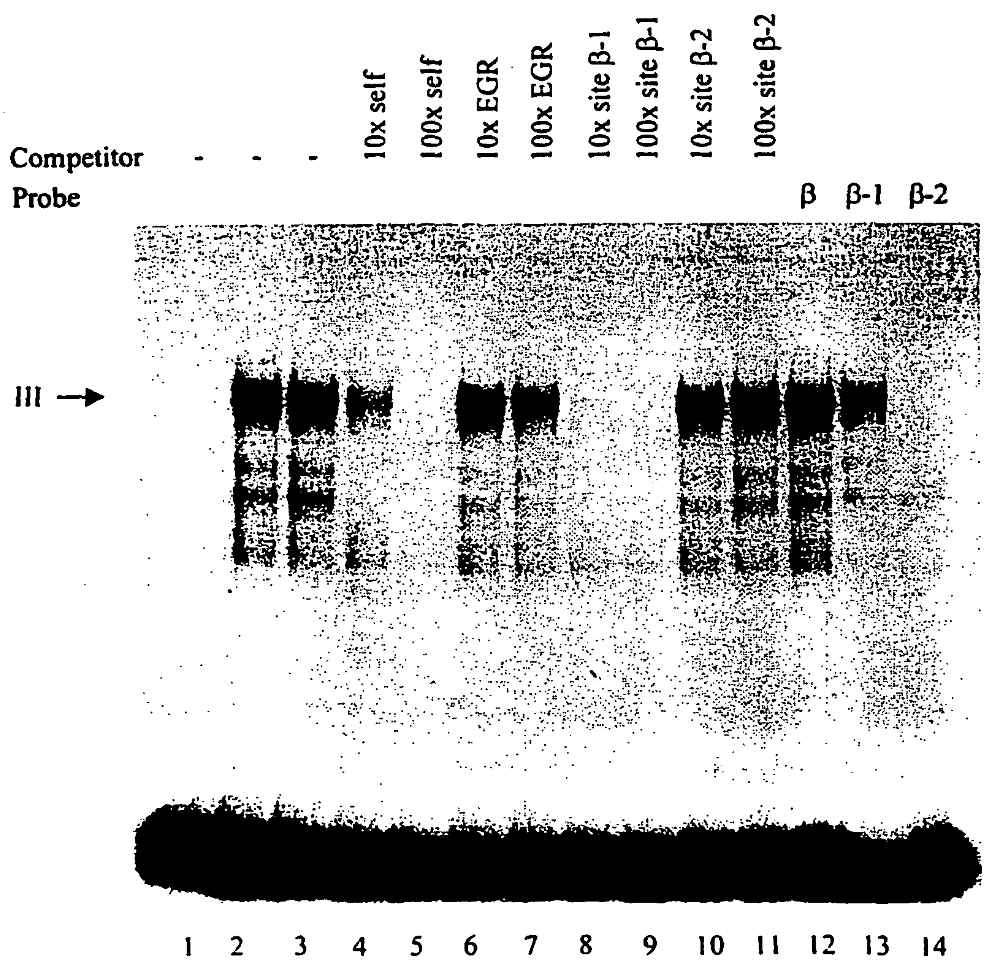


FIG. 9a.

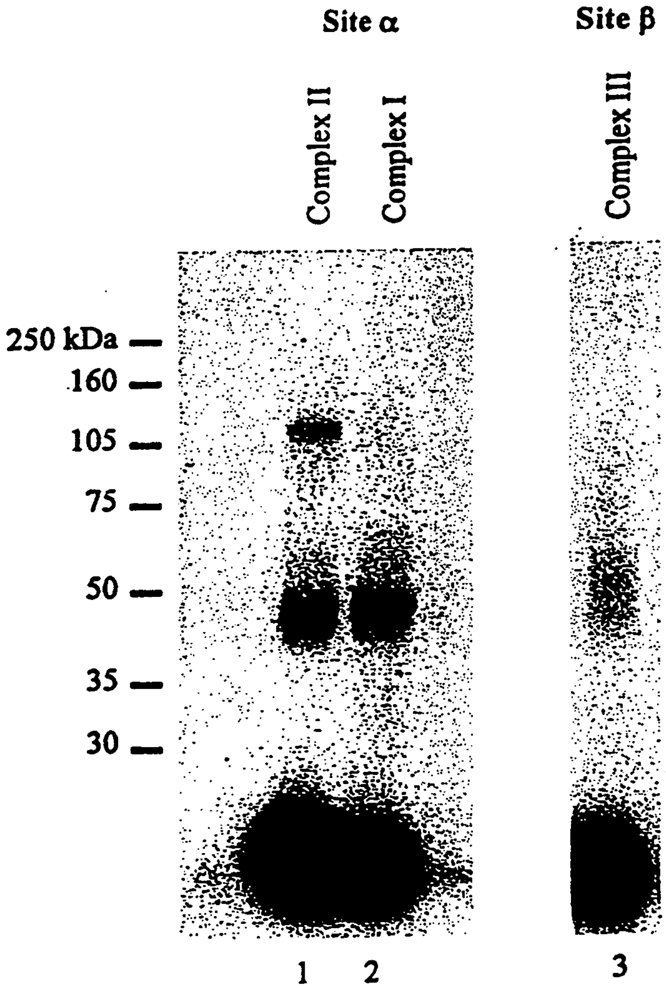


FIG. 9b.

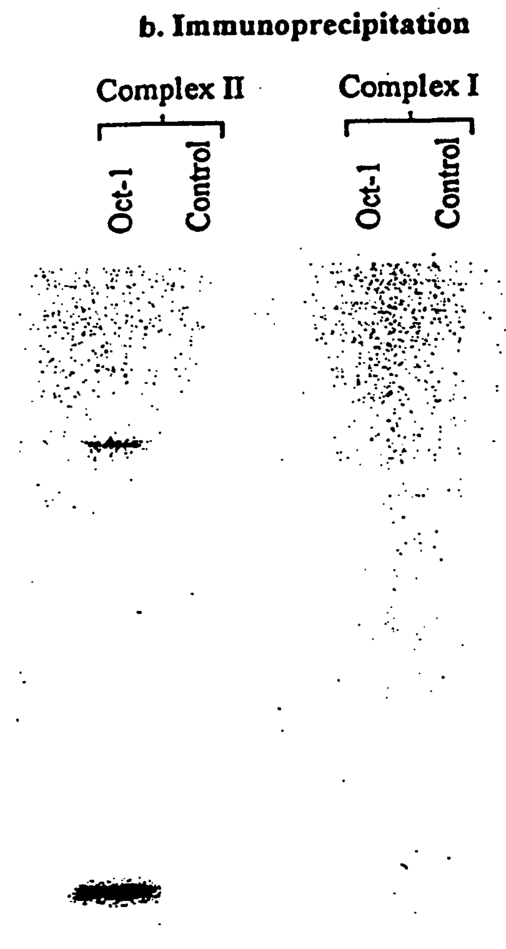


FIG. 10.

Site α_G	agctGTTCTATCTTTQTCCQGCATCCQGTCQGGAAGQTA agctQAACQQCCAGACAGGAQGCAGGAAAAAGAQAGAAC
Site α_A	agctGTTCTATCTTTQTCCQGCATCCQGTCQGGAAAQTA agctQAAQTQCCAGACAGGAQGCAGGAAAAAGAQAGAAC
Site β	agctQAGAAGGAAACAGACCACAGACCQG agctCAGGTCTGQGGQCTGQTQCCQTCTA

FIG. 11.

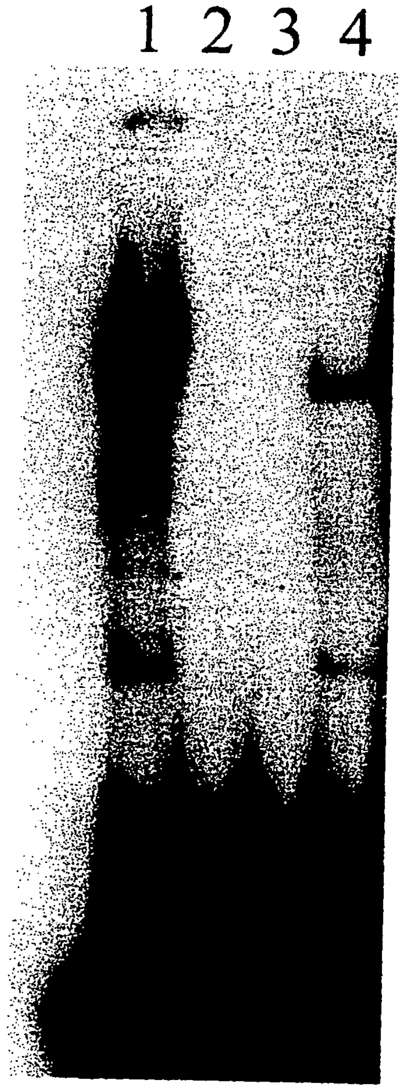
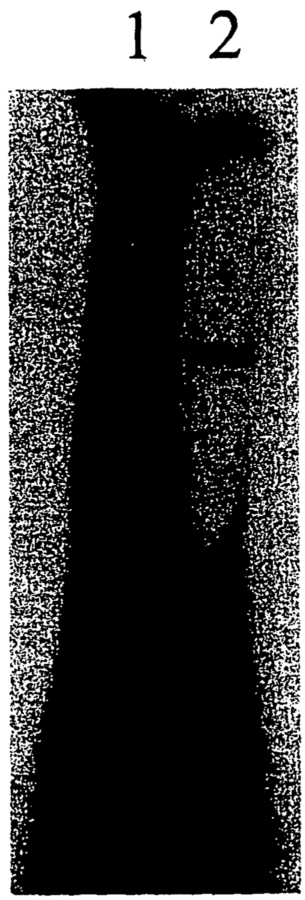


FIG. 12.



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00414

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C07K14/47 A61K35/00 C12N5/10 C12N15/85

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BRINKMAN B M ET AL: "Relevance of the tumor necrosis factor alpha (TNF alpha) -308 promoter polymorphism in TNF alpha gene regulation ‘see comments!." JOURNAL OF INFLAMMATION, (1995-96) 46 (1) 32-41. , XP000907438 the whole document	33-36
X	WO 97 42820 A (UNIV DUKE) 20 November 1997 (1997-11-20) the whole document	22
A	UDALOVA I A ET AL: "Complex NF-kappaB interactions at the distal tumor necrosis factor promoter region in human monocytes." JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 AUG 14) 273 (33) 21178-86. , XP002139037	1-36

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 May 2000	15/06/2000

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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